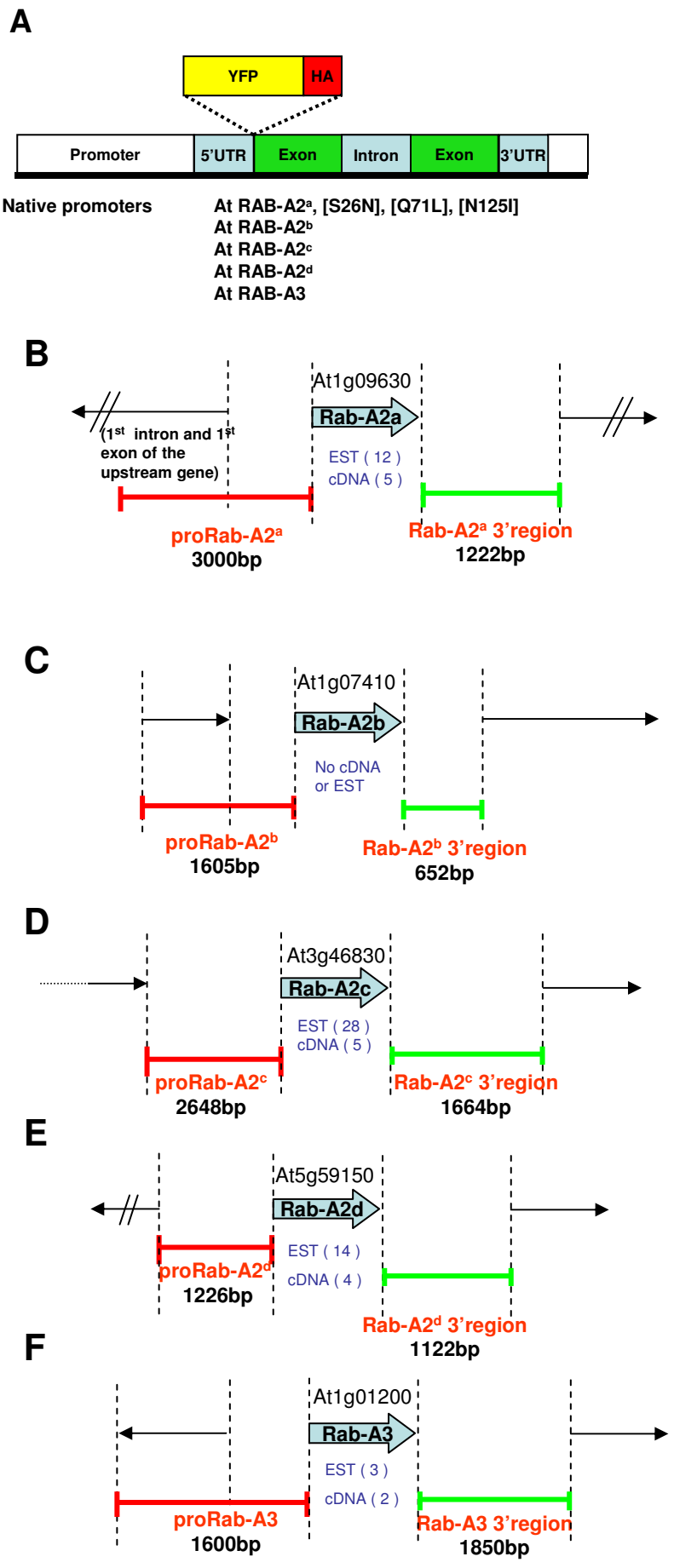
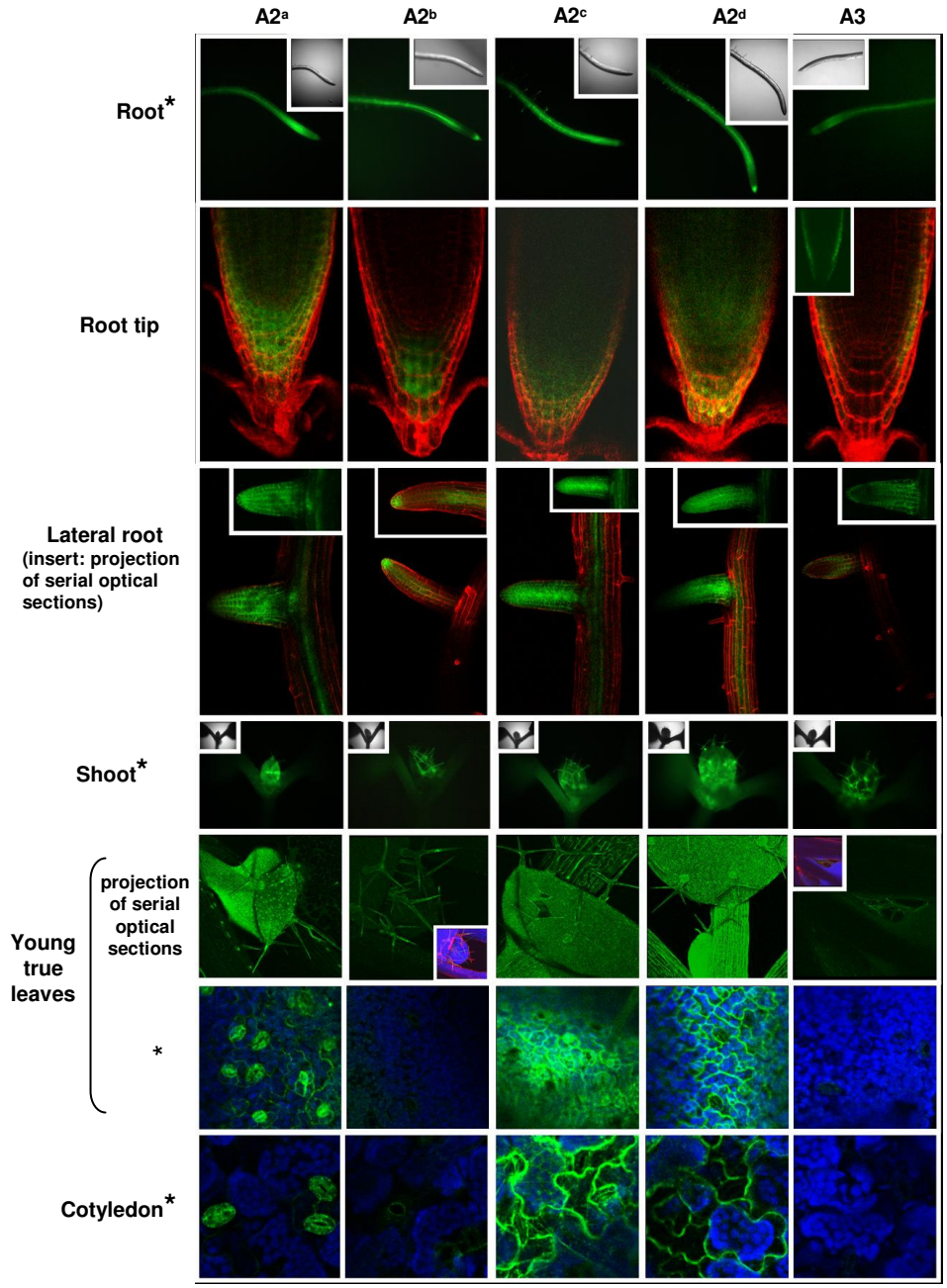
Supplemental Data. Chow et al., (2008) TITLE Rab-A2 and Rab-A3 GTPases define a *trans*-Golgi endosomal membrane domain in *Arabidopsis* that contributes substantially to the cell plate



Supplemental Figure 1. Genomic DNA fragments of Rab-A genes

A. Schematic diagram of the expression cassette of YFP:RAB-A fusions. The YFP-HA tag sequence was cloned in frame with the start codon of each Rab-A gene to generate an N-terminal YFP fusion protein. Non-transcribed intergenic regions are white.

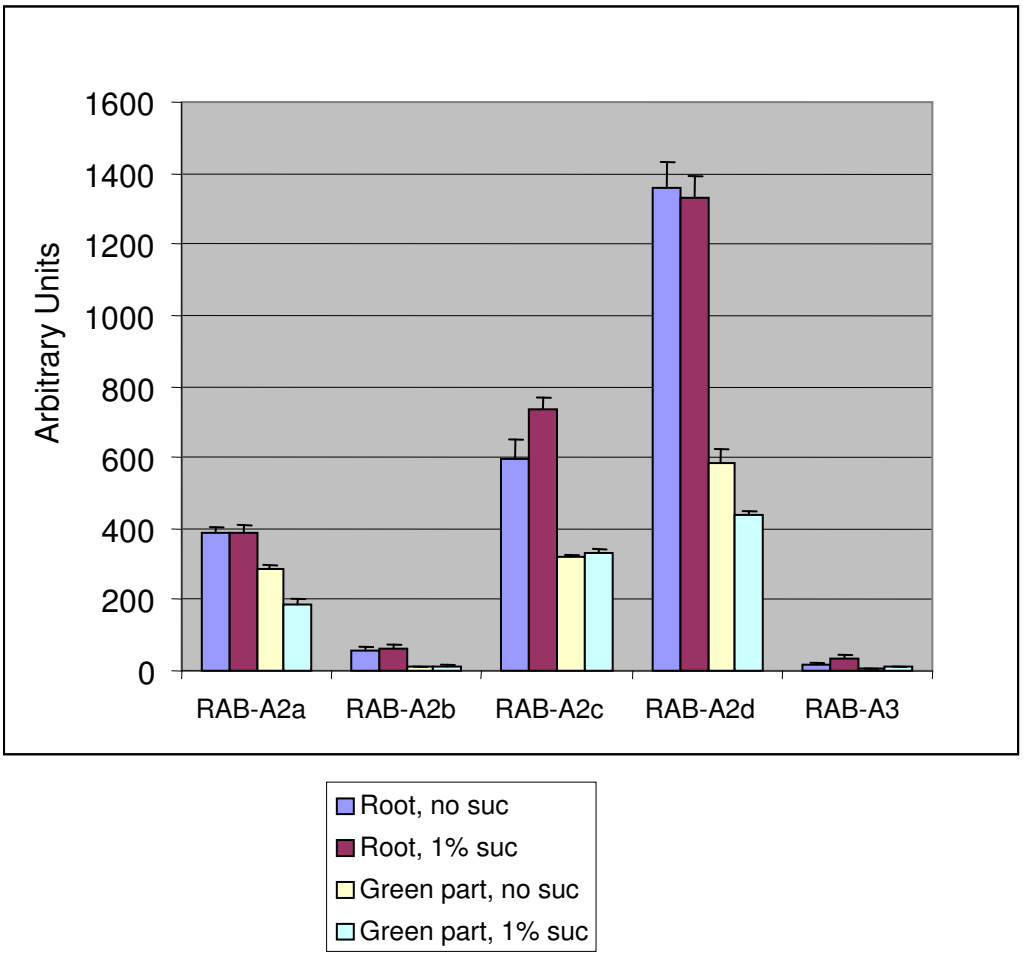
B-F. Schematic diagrams showing the At *RAB-A* genes and their 5' and 3' regions in the genome. Each YFP:RAB-A fusion included the coding region of At *RAB-A* gene (blue arrows; from the start codon to the stop codon), the promoter region (proRab-A, red line) and the 3' region (Rab-A 3' region, green line). The expression of all these genes except At *RAB-A2*^{b} is supported by the presence of EST and/or cDNA. Black arrows: adjacent genes. Blue characters: numbers of reported EST and cDNAs of the corresponding genes. Information was obtained from TAIR sequence viewer (www.arabidopsis.org).



*: imaging conditions identical for each line

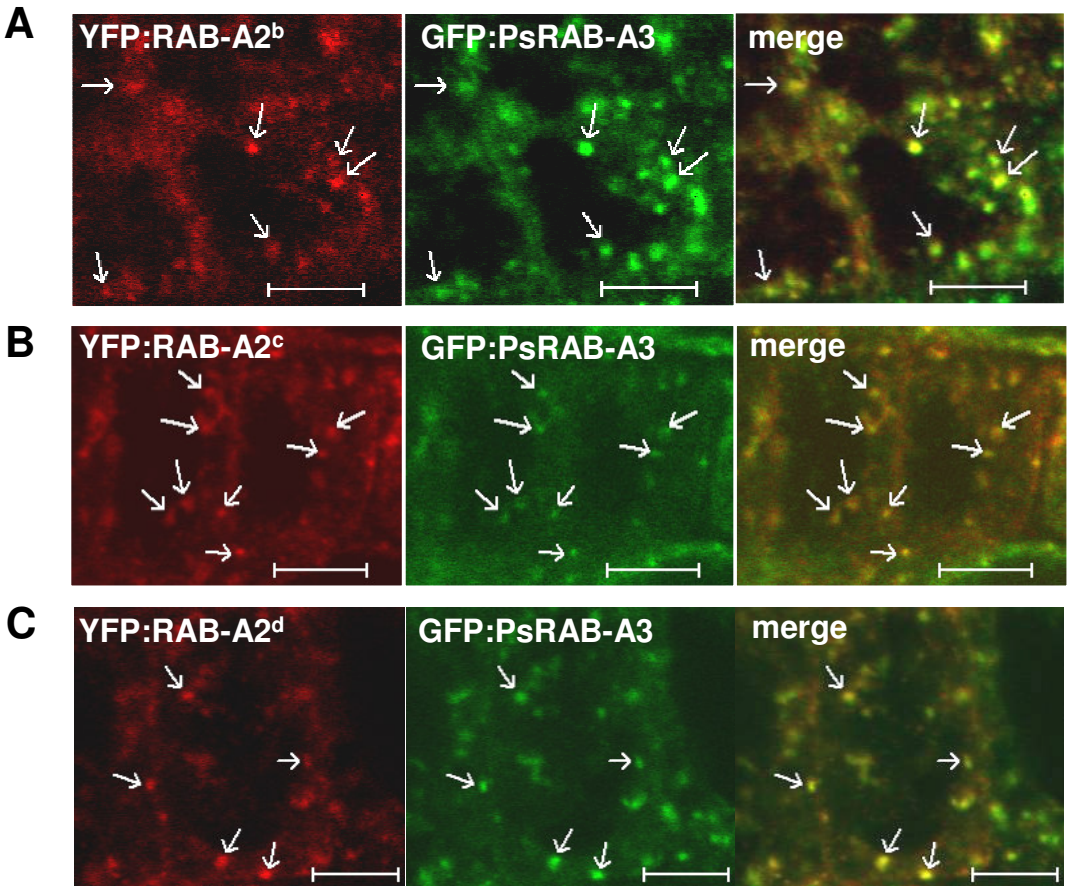
Supplemental Figure 2. YFP-fluorescence of YFP tagged genomic fusions of all members of the *Arabidopsis* Rab-A2 and Rab-A3 group

YFP-fluorescence of young seedlings expressing YFP:RAB-A fusions. 8-12-day old *Arabidopsis* seedlings grown on agar plates were examined by epifluorescence microscopy (1st and 4th rows, with brightfield images inset) and CLSM (the rest). Green: YFP. Red: propidium iodide (cell wall). Blue: auto-fluorescence of chlorophyll. Asterisks: images within these rows were taken under the same settings and thus direct comparison of intensities between lines is possible.



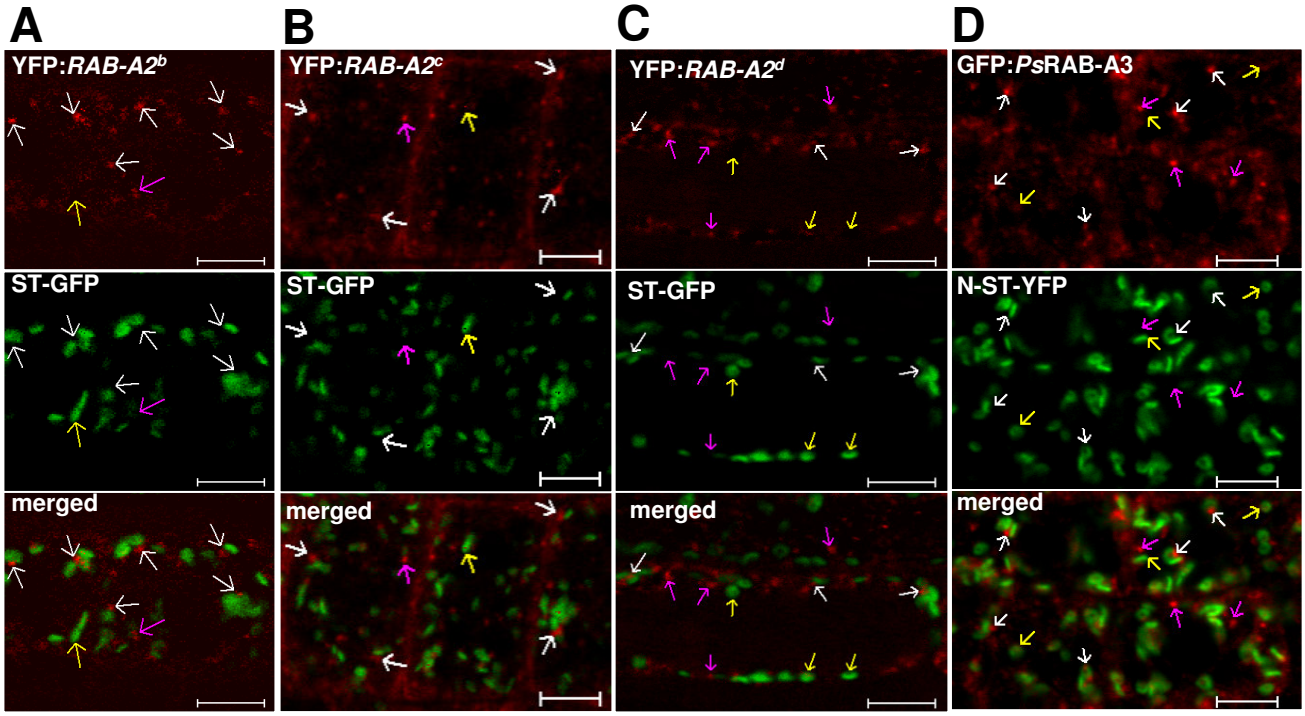
Supplemental Figure 3. Histograms of expression levels of members of the Rab-A2 and -A3 subclasses in *A. thaliana* plants extracted from the developmental data sets of AtGeneExpress (Schmidt et al., 2005).

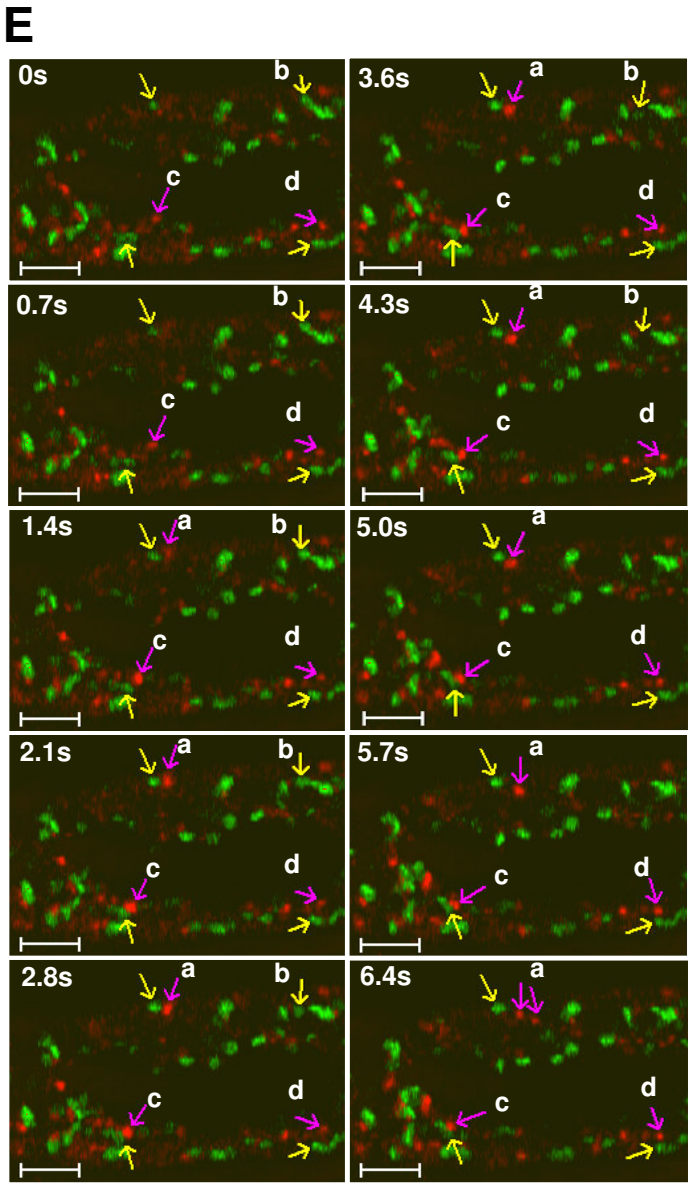
8 day-old seedling grown on MS medium under continuous light. Error bar: standard deviation n=3. 1% Suc = seedlings grown on sucrose; no suc = seedlings grown without sucrose.



Supplemental Figure 4. *At*RAB-A2 and *Ps*RAB-A3 label the same compartment.

CLSM analysis of seedling root-tip epidermis co-expressing GFP:*Ps*RAB-A3 (green) and YFP:RAB-A2^{b}, YFP:RAB-A2^{c} or YFP:RAB-A2^{d} as indicated (each red). White arrows: examples of structures with extensive overlap of GFP and YFP signals. Scale bar: 5µm





Supplemental Figure 5. xFP:RAB-A labelled compartments are distinct from the Golgi. CLSM analysis of seedling root cells co-expressing YFP/GFP:RAB-A fusions (red) with ST-GFP/YFP (green) as indicated. Epidermal cells at the root tip region are shown.

A-D. Yellow arrows indicate ST-xFP labelled Golgi stacks which are not close to any xFP:RAB-A-labelled structure; pink arrows indicate xFP:RAB-A labelled punctate structures which are not close to any ST-xFP labelled Golgi; white arrows indicate xFP:RAB-A labelled punctate structures which are closely associated with ST-xFP labelled punctate structures. Scale bars: 5µm.

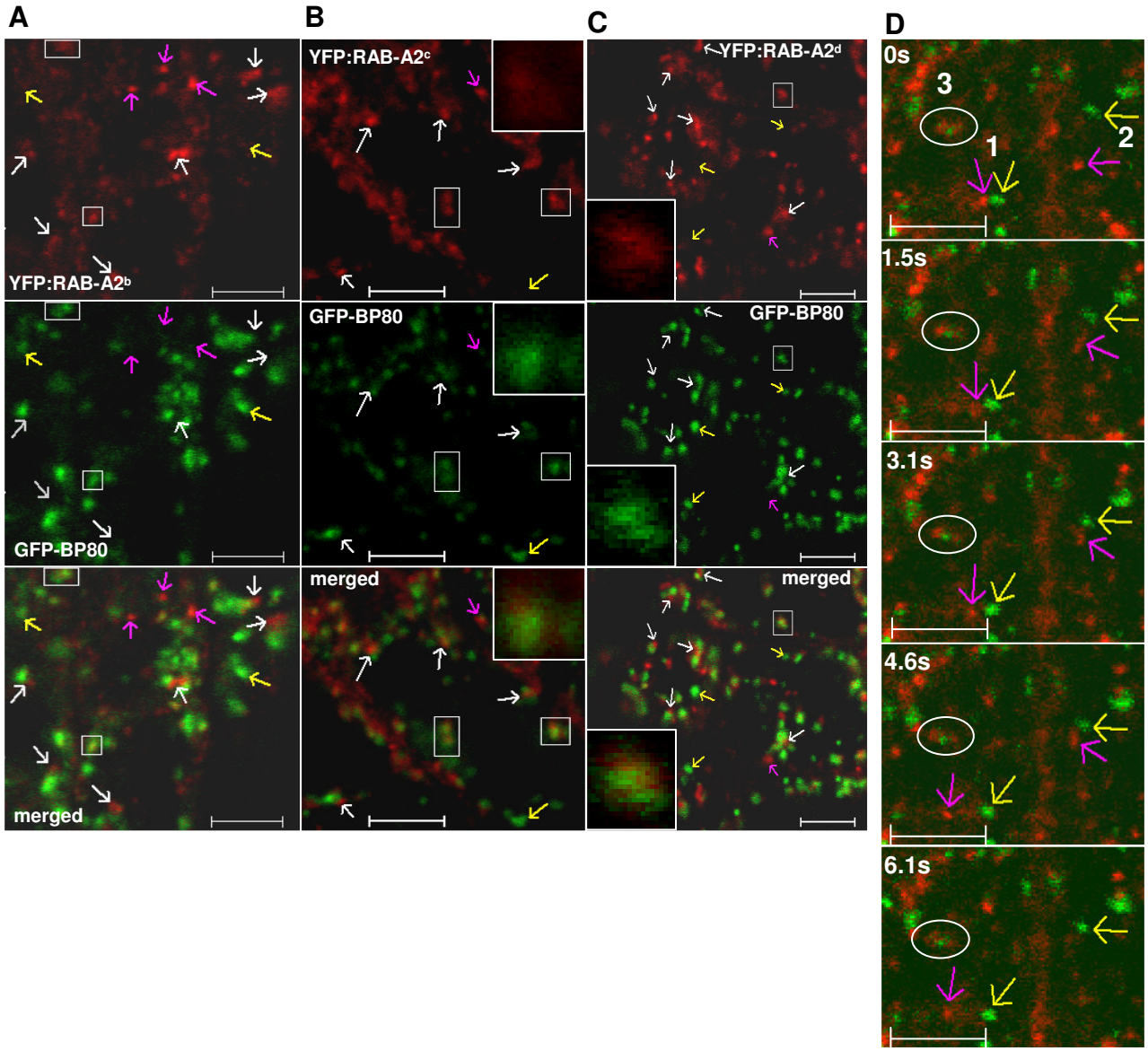
E. Time series of cells co-expressing YFP:RAB-A2^{a} (red) and ST-GFP (green). Lettered yellow or pink arrows respectively indicate Golgi or YFP:RAB-A2^{a}-labelled structures described below.

(a) a YFP-labelled puncta appears adjacent to a Golgi stack at 1.4 s but moves away at 5.7s. It possibly resolves into two smaller punctae at 6.4 s.

(b) a Golgi stack does not associate with any YFP-labelled structure before moving away from the focal plane.

(c) a YFP:Rab-A2^{a} structure moves towards a Golgi from 0s to 1.4 s and these two structures stay together till the end of the series.

(d) a YFP:Rab-A2^{a} structure appears to be getting closer to a Golgi from 3.6s to 5.7 s.

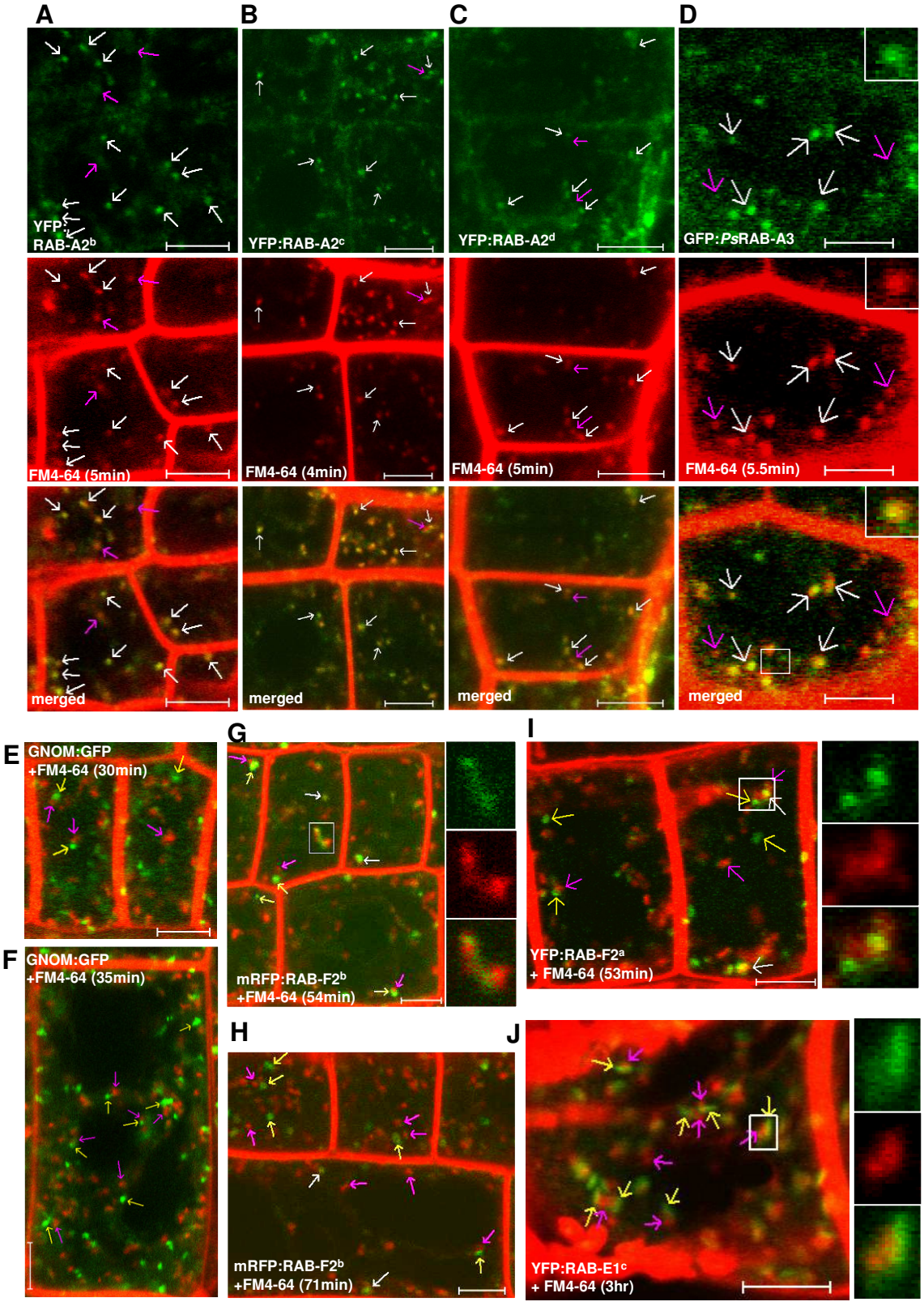


Supplemental Figure 6. YFP:RAB-A labelled compartments are distinct from the GFP-BP80 labelled PVC.

CLSM analysis of seedling root cells co-expressing YFP:RAB-A proteins as indicated (red) and GFP-BP80 (green).

A-C. Yellow arrows indicate GFP-BP80 labelled structures that are not close to any YFP:RAB-A labelled structure; pink arrows indicate YFP:RAB-A labelled punctate structures that are not close to any GFP-BP80 labelled structure; white arrows indicate YFP:RAB-A labelled puncta that are closely associated with GFP-BP80 structures. Boxed regions: overlap of YFP:RAB-A and GFP-BP80. Inserts: enlarged images of some boxed regions. Scale bars: 5µm.

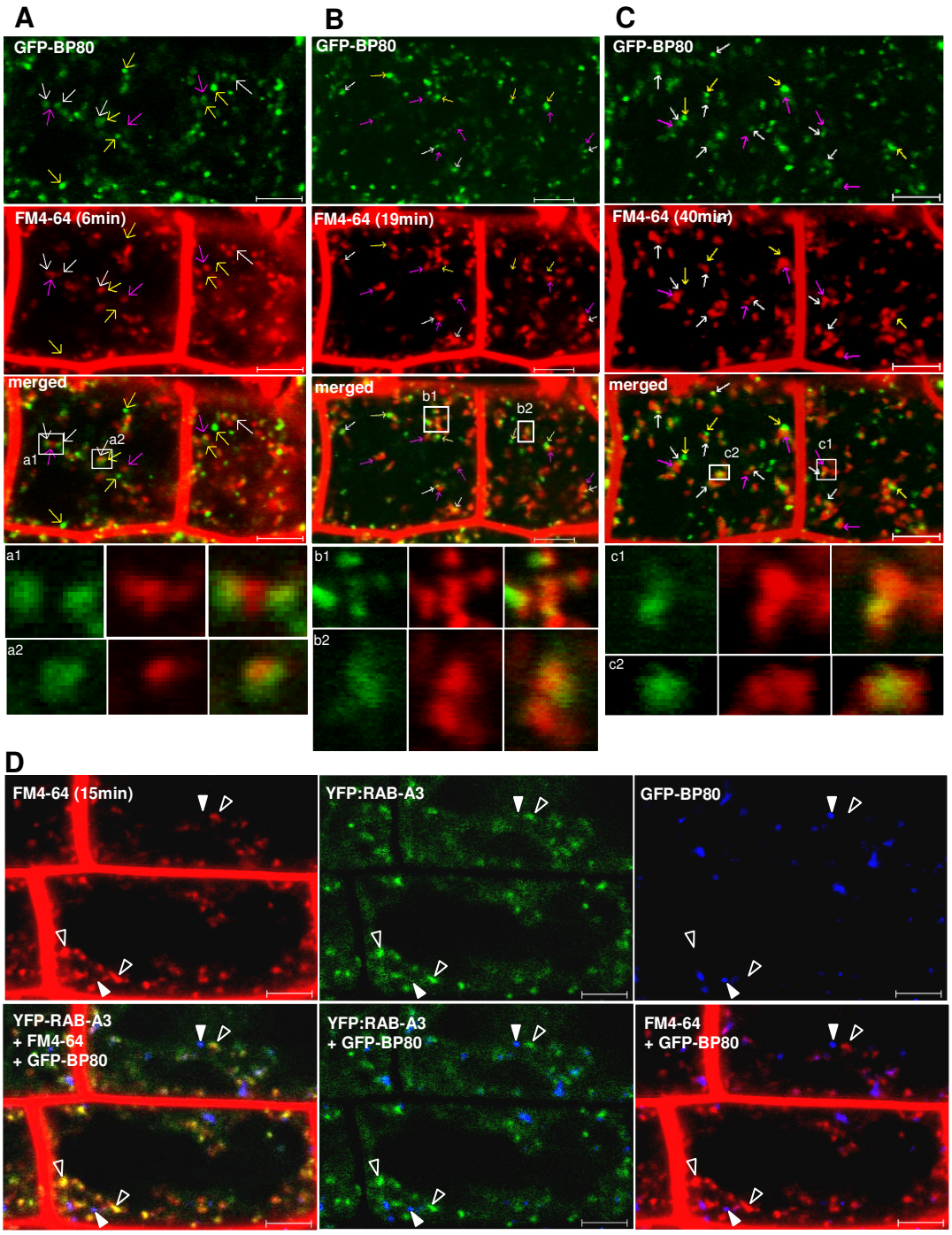
D. Time series from cell expressing YFP:RAB-A2^{d} (red) and GFP-BP80 (green). Green and red structures can either move apart (1), move closer (2), or remain closely associated (3). Scale bars: 5µm



Supplemental Figure 7 xFP:*At*RAB-A labelled compartments are rapidly labelled by FM4-64 while compartments labelled by xFP fusions to *At*RAB-E1^c, *At*RAB-F2 or GNOM are labelled inefficiently by FM4-64.

CLSM analysis of epidermal cells at the tip of seedling roots expressing YFP:RAB-A2^b (**A**), YFP:RAB-A2^c (**B**) YFP:RAB-A2^d (**C**), GFP:*Ps*RAB-A3 (**D**), GNOM:GFP (**E-F**), mRFP:RAB-F2^b (**G-H**), YFP:RAB-AF2^a (**I**) or YFP:RAB-E1^c (**J**) incubated with FM4-64 for the indicated time. Colocalisation of FM4-64 in an xFP labelled structure is rare even after long incubation in **E-J** in contrast to the rapid colocalisation between xFP:RAB-A and FM4-64 (**A-D**). Red channel: FM4-64. Green channel: xFP. Yellow arrows: xFP labelled regions without detectable FM4-64 labelling. Pink arrows: FM4-64 labelled regions without detectable xFP signal. White arrows: regions with extensive overlap of xFP and FM4-64 signals. Enlarged images of boxed area are presented as inserts or separate columns.

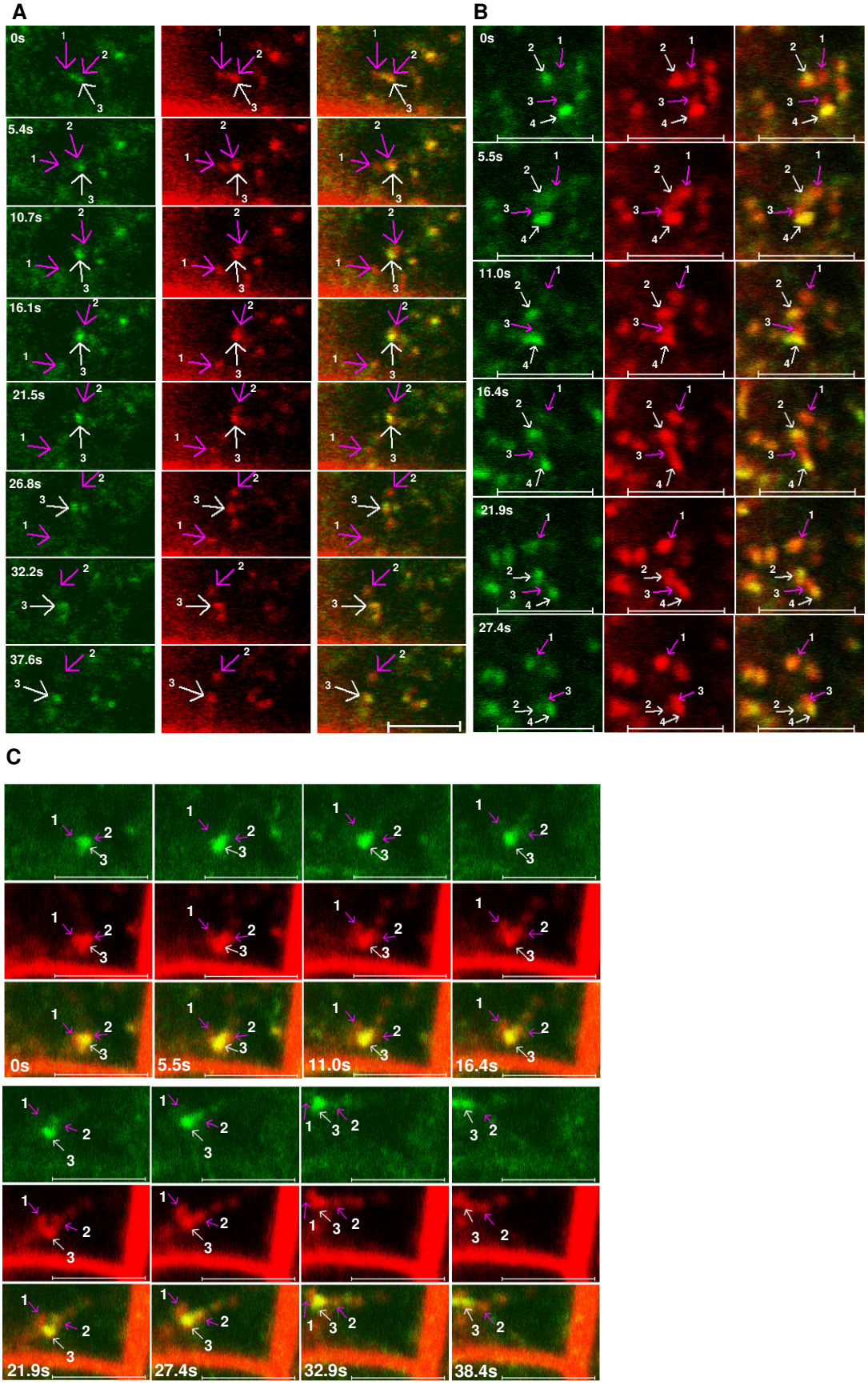
Scale bars = 5µm



Supplemental Figure 8. FM4-64 labels GFP-BP80 compartments less efficiently than the YFP:RAB-A2/3 compartment.

A-C: CLSM analysis of root tip epidermal cells of seedlings expressing GFP-BP80 incubated with FM4-64 for the indicated times. Most GFP-BP80 labelled compartments lack FM4-64 labelling. A minority of GFP-BP80-labelled structures partially overlap with FM4-64 (for examples, boxed region). Yellow arrows: GFP labelled regions without detectable FM4-64 labelling. Pink arrows: FM4-64 labelled regions without detectable GFP signal. White arrows: region with extensive overlap of GFP and FM4-64 signals. Enlargements of boxed areas are presented below each image.

D: CLSM analysis of root tip epidermal cells of seedlings co-expressing YFP:RAB-A3 (green) and GFP-BP80 (blue) incubated with FM4-64 (red) for 15 minutes. YFP:RAB-A3 but not GFP-BP80 labelled compartments are extensively co-labelled with FM4-64. Top row, single channels; bottom row overlay of two or three channels as indicated. Open arrowheads: YFP:RAB-A3. Arrowheads: GFP-BP80. Scale bars: 5µm.


A
0s
5.4s
10.7s
16.1s
21.5s
26.8s
32.2s
37.6s
B
0s
5.5s
11.0s
16.4s
21.9s
27.4s
C
0s
5.5s
11.0s
16.4s
21.9s
27.4s
32.9s
38.4s

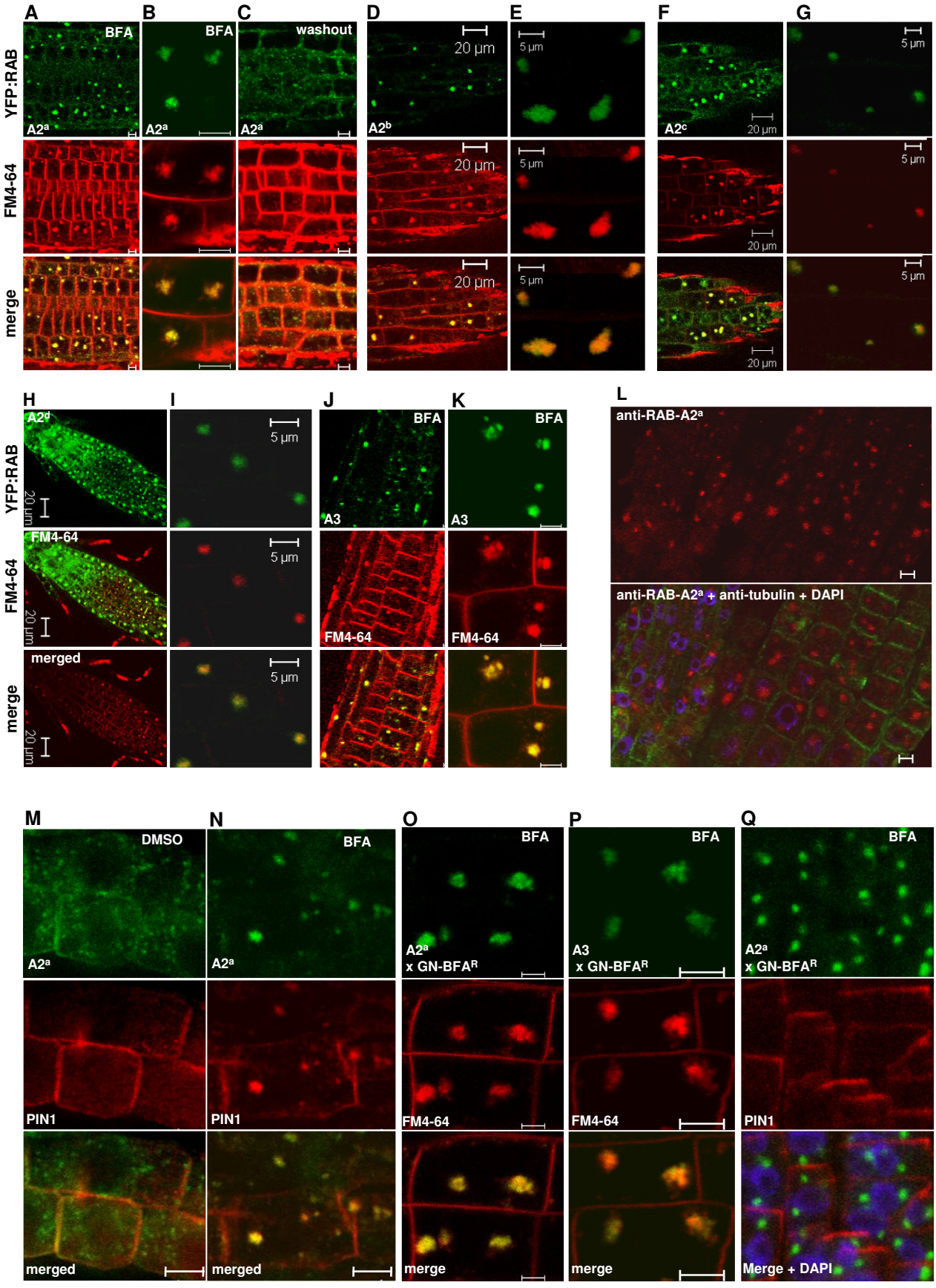
Supplemental Figure 9. Time series analysis of FM4-64 labelling of the Rab-A2/A3 compartment.

Time lapse CLSM images of seedling roots expressing YFP:RAB-A2^{d} (A) or GFP:*Ps*RAB-A3 (B and C) (each shown in green) incubated with FM4-64 (red) for 10min. Pink arrows: FM4-64 labelled regions without detectable YFP/GFP signal. White arrows: region with extensive overlap of YFP/GFP and FM4-64 signals.

A: At the start of the time series, there is a red (FM4-64) punctate structure partly labelled by green signal (YFP:RAB-A2^{d}) (white arrow). At the subsequent time points, regions enriched in FM4-64 alone (1 and 2) separate out as distinct punctate structures. Part of the structure stays yellow over 30s (3), suggesting that the overlap was a case of colocalisation.

B: Compartments 1 and 2 move together and apparently form parts of a single compartment with regions 3 and 4 at 5.5s but resolve into two distinct structures from 11.0s. Compartment 1 remains distinct and FM4-64-enriched but appears to acquire increasing GFP:*Ps*RAB-A3 signal over the next 16 seconds. Compartment 2 re-associates with regions 3 and 4 from16.4s-27.4s apparently forming a single elongated structure. Note that FM4-64 enriched region 3 and colocalised region 4 remain together throughout the time series.

C: The FM4-64 enriched region (1) appears to dissociate from the colocalised yellow region (3) throughout the time series and a second FM4-64 enriched region appears nearby at 38.4s (2).


A
B
C
D
E
F
G
YFP:RAB
FM4-64
merge
BFA
BFA
washout
A2a
A2a
A2a
A2b
A2c
20 µm
5 µm
H
I
J
K
L
A2d
A3
A3
anti-RAB-A2a
anti-RAB-A2a + anti-tubulin + DAPI
merged
M
N
O
P
Q
DMSO
BFA
A2a
x GN-BFAR
A3
x GN-BFAR
PIN1
FM4-64
merge
Merge + DAPI

Supplemental Figure 10. Rapid relocation of YFP:RAB-A2 and -A3 to BFA bodies in *Arabidopsis* root tips.

CLSM analysis of seedling roots. Seedlings were pre-incubated with FM4-64 for 10-30 minutes before BFA treatment (25µM BFA for 30-60 minutes). All four YFP:RAB-A2 fusions as well as YFP:RAB-A3 relocated almost completely to the BFA body where they colocalised precisely with internalised FM4-64 or the recycling plasma-membrane protein AtPIN1

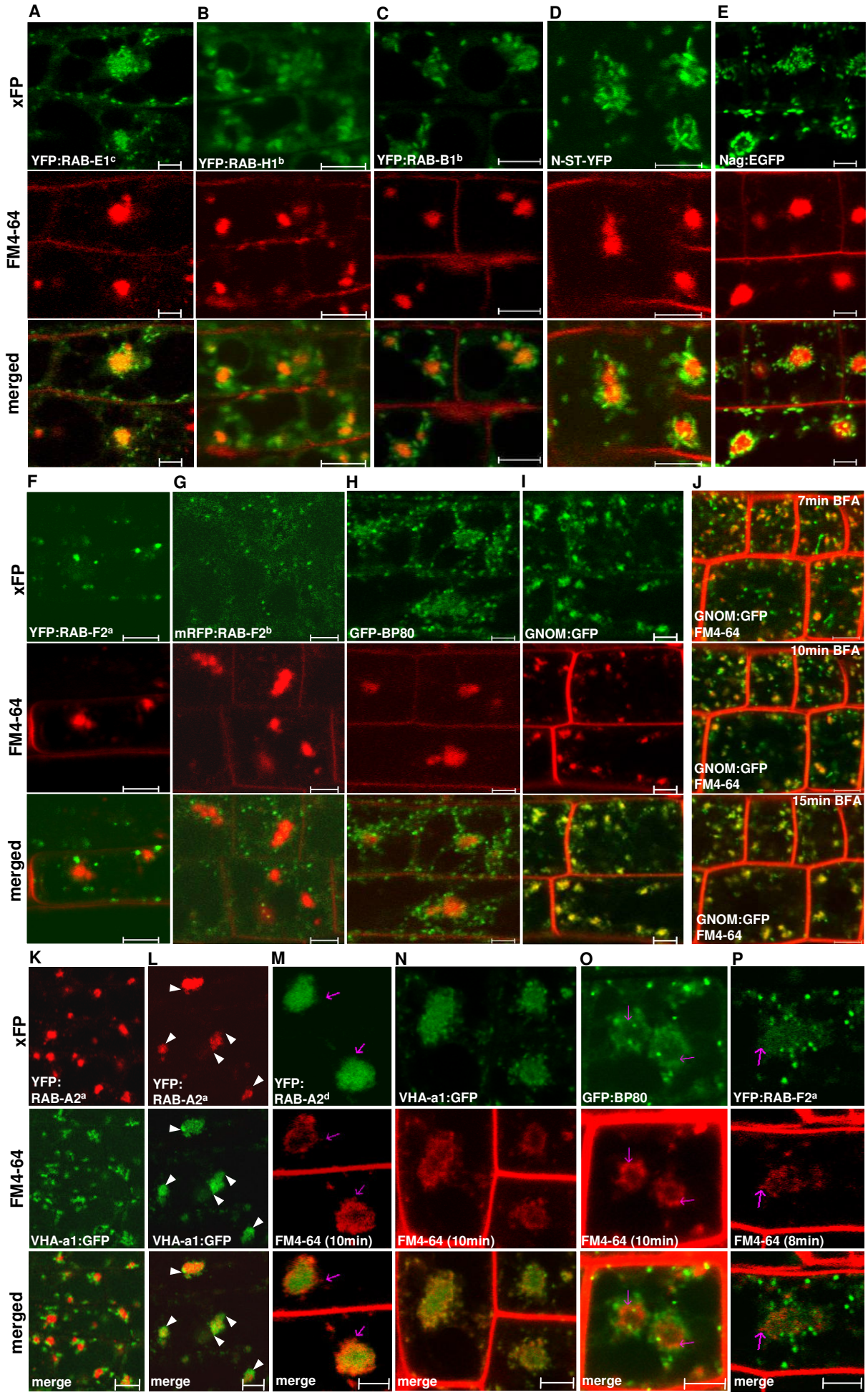
A-K YFP:RAB-A fusions are green and FM4-64 is in red at low magnification (A, C, D, F, H, J; bars 20µm) or high magnification (B,E,G,I,K; bars 5µm). YFP:RAB-A2^{a} seedlings recovered from BFA treatment after being washed and incubated in MS solution (C).

L. Endogenous *At*RAB-A2^{a} localises to the BFA-compartments. Double immunolocalisation of alpha-tubulin (green) and *At*RAB-A2^{a} (red) in wild-type roots stained with DAPI (blue)**.** 5 day-old seedlings were treated with BFA (50µM for 1 hour) before fixation. Affinity-purified anti-RAB-A2^{a} (1:3000) was used to detect the native *At*RAB-A2^{a} proteins.

M, N. Immunolocalisation of PIN1 (red) after treatment with BFA (50µM BFA for 1hour) (N) or DMSO (M). PIN1 and YFP:RAB-A2^{a} colocalised in BFA bodies upon BFA treatment.

O-Q. In the presence of BFA-resistant GNOM [M696L] (GN-BFA^{R}) which is sufficient for recycling PIN1 to the plasma membrane in the presence of BFA, YFP:RAB-A2^{a} (O, Q) and YFP:RAB-A3 (P) still relocated to BFA-bodies and colabelled with FM4-64 in BFA bodies whereas PIN1 maintained its polarized PM localisation showing that GNOM [M696L] was functionally expressed (Q; blue shows DAPI-labelled nuclei). Thus GNOM activity is not sufficient to keep Rab-A2/A3 membranes from accumulating in the BFA body.

Scale bars = 5µm unless indicated


A
B
C
D
E
xFP
FM4-64
merged
YFP:RAB-E1c
YFP:RAB-H1b
YFP:RAB-B1b
N-ST-YFP
Nag:EGFP
F
G
H
I
J
YFP:RAB-F2a
mRFP:RAB-F2b
GFP-BP80
GNOM:GFP
7min BFA
GNOM:GFP
FM4-64
10min BFA
GNOM:GFP
FM4-64
15min BFA
GNOM:GFP
FM4-64
K
L
M
N
O
P
YFP:
RAB-A2a
YFP:
RAB-A2a
YFP:
RAB-A2d
VHA-a1:GFP
GFP:BP80
YFP:RAB-F2a
VHA-a1:GFP
VHA-a1:GFP
FM4-64 (10min)
FM4-64 (10min)
FM4-64 (10min)
FM4-64 (8min)
merge
merge
merge
merge
merge
merge

Supplemental Figure 11. Subcellular compartments of *Arabidopsis* root tip epidermal cells respond differently to BFA treatment.

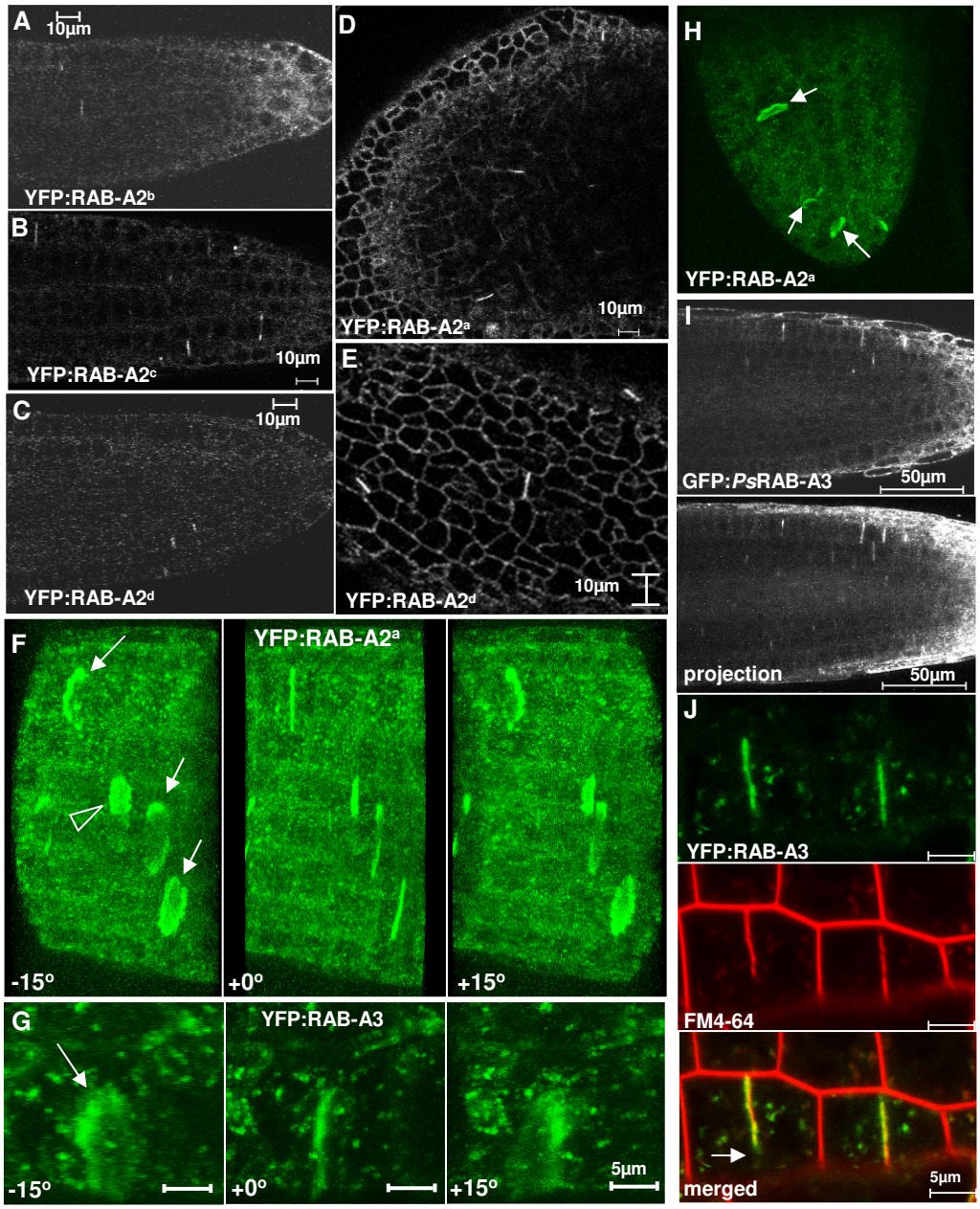
A-H. CLSM analysis of seedlings pre-incubated with FM4-64 for 10-40 minutes before BFA treatment (25µM BFA for 30-60 minutes). Green: xFP. Red: FM4-64. xFP:RAB fusions to members of Rab-B, Rab-E, Rab-H subclasses (A-C) which are localised mainly to the Golgi and the Rab-F2 subclass that localises to the PVC (F and G) remained principally associated with discrete punctate structures that either surrounded BFA bodies or were dispersed in the cytoplasm; in addition, they also faintly labelled the core of the BFA bodies. The integral Golgi markers N-ST-YFP and Nag:EGFP (D and E) and the integral PVC marker GFP-BP80 also mainly localized to multiple discrete, punctate structures surrounding the BFA bodies though faint GFP-BP80 signal was also associated with the core of the BFA bodies.

I-J. BFA treatment causes the GNOM:GFP (green) compartment to colocalise with FM4-64 (red) labelled compartments in the forming BFA-bodies in the elongation zone (I) and meristem (J) and elongation zone of roots (B). Colocalisation within forming BFA bodies is observed within 7 minutes of BFA application and was complete by 15 minutes (B).

K and L. CLSM analysis of young seedlings expressing YFP:RAB-A2^{a} and VHA-a1:GFP after addition of BFA. YFP:RAB-A2^{a} relocates to the forming BFA bodies more quickly than VHA-a1:GFP and there is little co-localisation between the markers (K). At later stages (L), regions enriched in VHA-a1 can be observed at the periphery of the BFA bodies (arrowheads). Thus the partial overlap in the distribution of these two markers appeared to be preserved in the BFA compartment. This suggests that some degree of membrane identity and organisation is maintained within the BFA-body.

M-P. CLSM analysis of FM4-64 uptake into seedling root tips pretreated with 50µM BFA for 40 minutes. Times in the brackets (middle row) are the incubation times with FM4-64 and BFA after the BFA pre-treatment. Seedlings expressing YFP:RAB-A2^{d} (M), VHA-a1:GFP (N), GFP-BP80 (O) or YFP:RAB-F2^{a} (P). FM4-64 first accumulates in a shell surrounding the Rab-A2/A3 membranes in the core of the BFA body and surrounded in turn by PVC markers (O and P) which are largely excluded from the core of the BFA body.

Scale bars = 5µm.



Supplemental Figure 12. RAB-A2/A3 target xFP to cell-plate-like structures which are rapidly labelled with FM4-64.

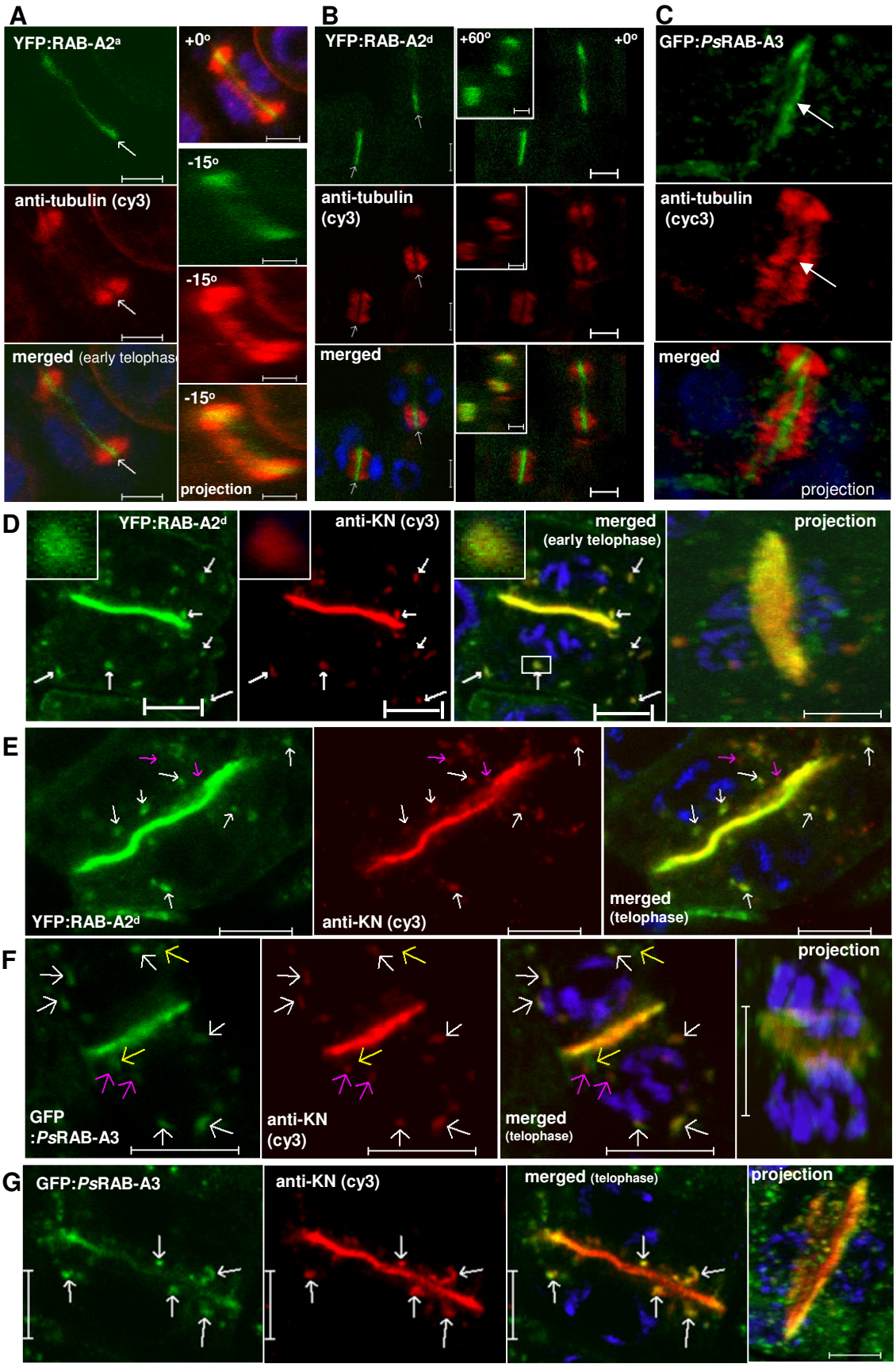
Meristemic tissues of root tips (A-C, F-J) or young true leaves (D-E) of *Arabidopsis* seedlings expressing fluorescent protein fusions to *At*Rab-A2^{b} (A), *At*Rab-A2^{a} (D, F, H), *At*Rab-A2^{c} (B), *At*Rab-A2^{d} (C,E), *At*Rab-A3 (G, J) or *Ps*Rab-A3 (I). xFP:RAB-A in white or green; FM4-64 in red.

A-E, I. xFP:RAB signals were seen to concentrate in linear structures in low magnification CLSM images. Single sections are shown unless specified.

F-H. Projections in *z* of meristemic regions of root tips**.** Arrows: Ring-shaped cell plates. Arrowhead: disc-shaped cell plate.

J. YFP:RAB-A3 labelled discs were colabelled by FM4-64. Note that one of the YFP and FM4-64 colabelled structures has an open end (arrows).

Scale bars: 5µm, 10µm or 50µm as indicated.

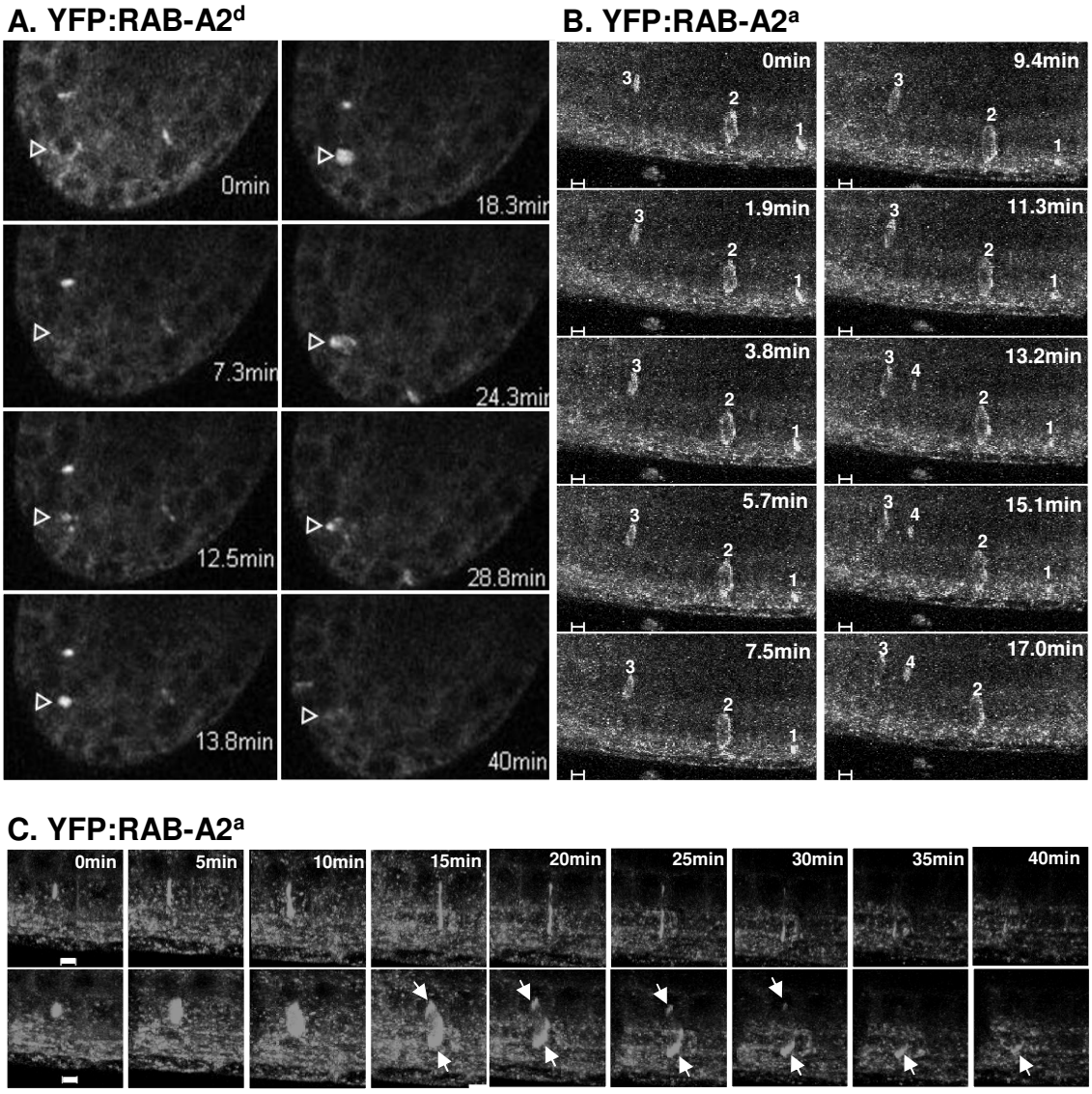

A
YFP:RAB-A2a
+0°
-15°
anti-tubulin (cy3)
-15°
merged (early telophas
-15°
projection
B
YFP:RAB-A2d
+60°
+0°
anti-tubulin (cy3)
merged
C
GFP:PsRAB-A3
anti-tubulin (cyc3)
merged
projection
D
YFP:RAB-A2d
anti-KN (cy3)
merged (early telophase)
projection
E
YFP:RAB-A2d
anti-KN (cy3)
merged (telophase)
F
GFP :PsRAB-A3
anti-KN (cy3)
merged (telophase)
projection
G
GFP:PsRAB-A3
anti-KN (cy3)
merged (telophase)
projection

Supplemental Figure 13. Tagged RAB-A2/A3 proteins localised to cell plates which were sandwiched by the phragmoplast and colabelled by KNOLLE (KN).

Meristemic tissues of root tips of 5-day-old *Arabidopsis* seedlings expressing fluorescent protein fusions to *At*RAB-A2^{a}(A), *At*RAB-A2^{d} (B,D-E) and *Ps*RAB-A3 (C, F-G). Single section CLSM images unless otherwise stated.

A-C. Immunolocalisation of alpha-tubulin in red; DAPI in blue; xFP in green. The right columns in A and B are 3-D reconstruction images of serial sections of the cell plates shown in the left columns. xFP signal is found in the mid-zone of the phragmoplast detected by anti-alpha-tubulin (arrows).

D-G. Immunolocalisation of KN. xFP:RAB-A in green; KNOLLE in red; DAPI in blue. White arrows: extensive overlap between xFP:RAB-A and KN signals. Yellow arrows: xFP:RAB-A signal alone. Pink arrows: KN signal alone. For D,F and G, *z*-projections of the merged channel are shown at the right hand side in addition to the single optical sections in the first three rows. Enlarged images of the boxed regions in D are shown as inserts. Scale bars: 5µm.



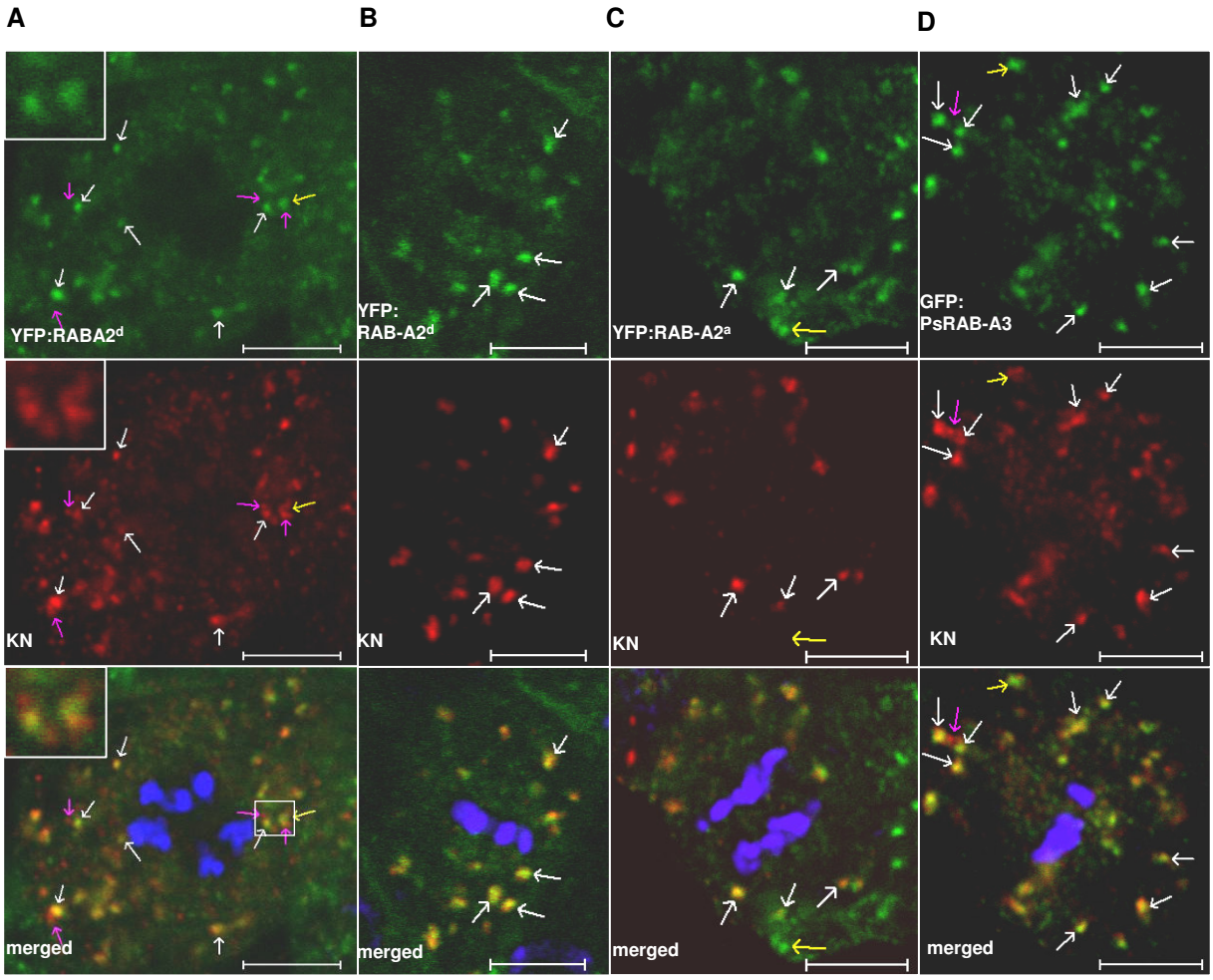
Supplemental Figure 14. Labelling patterns of YFP:RAB-A2 labelled cell plates changes over time as shown by the time series of 3D images reconstructed from serial sections.

CLSM analysis of *Arabidopsis* seedling roots expressing YFP:RAB-A (white). Scale bars: 5µm.

A. YFP:RAB-A2^{d}. Selected projections from a 4-D movie (Supplementary Movie S1). Arrowheads point to the cell where a cell plate appears, expands, concentrates at corners, and finally disappears. At 0 minutes the protein was located in the cytoplasm with the nuclear region clearly visible as a dark disc. This was lost by 7.3 minutes, corresponding perhaps to chromosome separation. By 12.5 minutes YFP:RAB-A2^{d} had begun to assemble into larger aggregates which fused together within 1.5 minutes to form a disc that expanded outwards (13.8-24.3 minutes) apparently dissipating as it reached the cell periphery. It thus persisted longest in the four corners (28.8 minutes) which would be the last points of a cuboid cell to be reached by an expanding central disc. By 40 minutes the distribution of YFP:RAB-A2^{d} resembled that at 0 minutes. In these cells, cell plate formation required approximately 20-30 minutes.

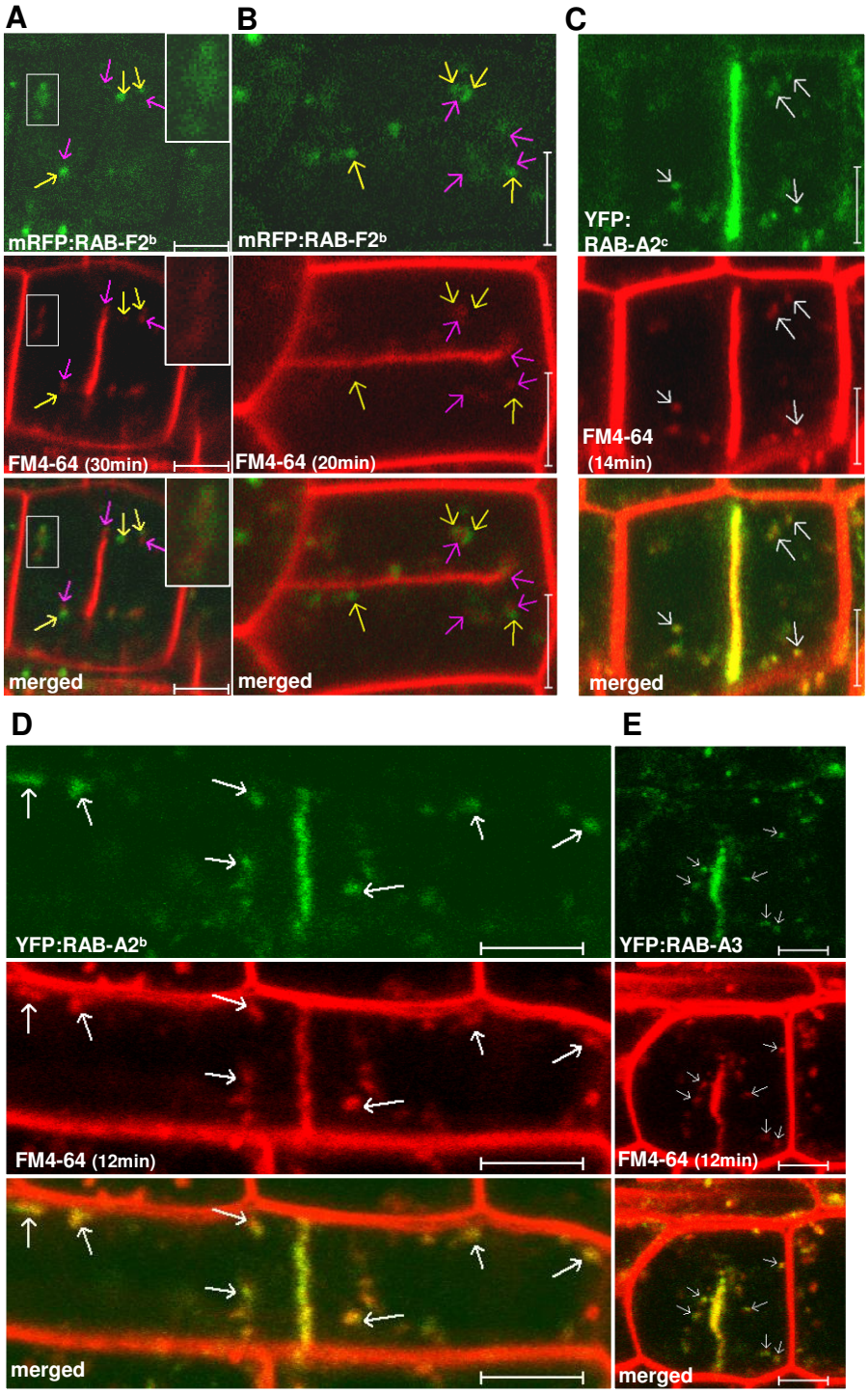
B. YFP:RAB-A2^{a}. Projections from a 4-D movie showing changes in four cell plates at different stages over 17 minutes. (1): a semi-circular structure gradually reduces in size and disappears near the end of the time series. (2): a ring-shaped structure expands and the YFP signal almost disappears from one side of the ring at the end of the time series. (3): a solid disc expands and transforms into a ring-shaped structure. (4): a small solid disc appears and expands.

C. YFP:RAB-A2^{a}. Projections of a cell plate at two different angles (upper and lower rows) over 40 minutes. The YFP signals becomes polarised at two ends (arrows) from 15 minute onwards.

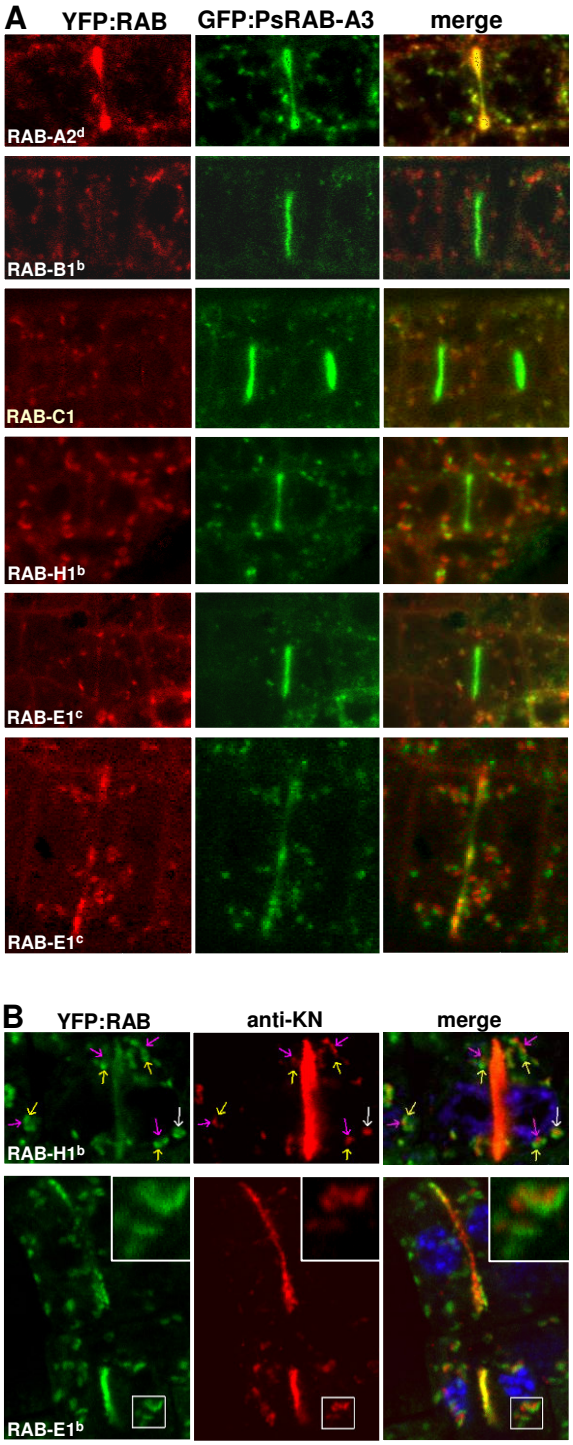


Supplemental Figure 15. xFP:RAB-A2/A3 localised to KN-positive structures before the onset of cytokinesis

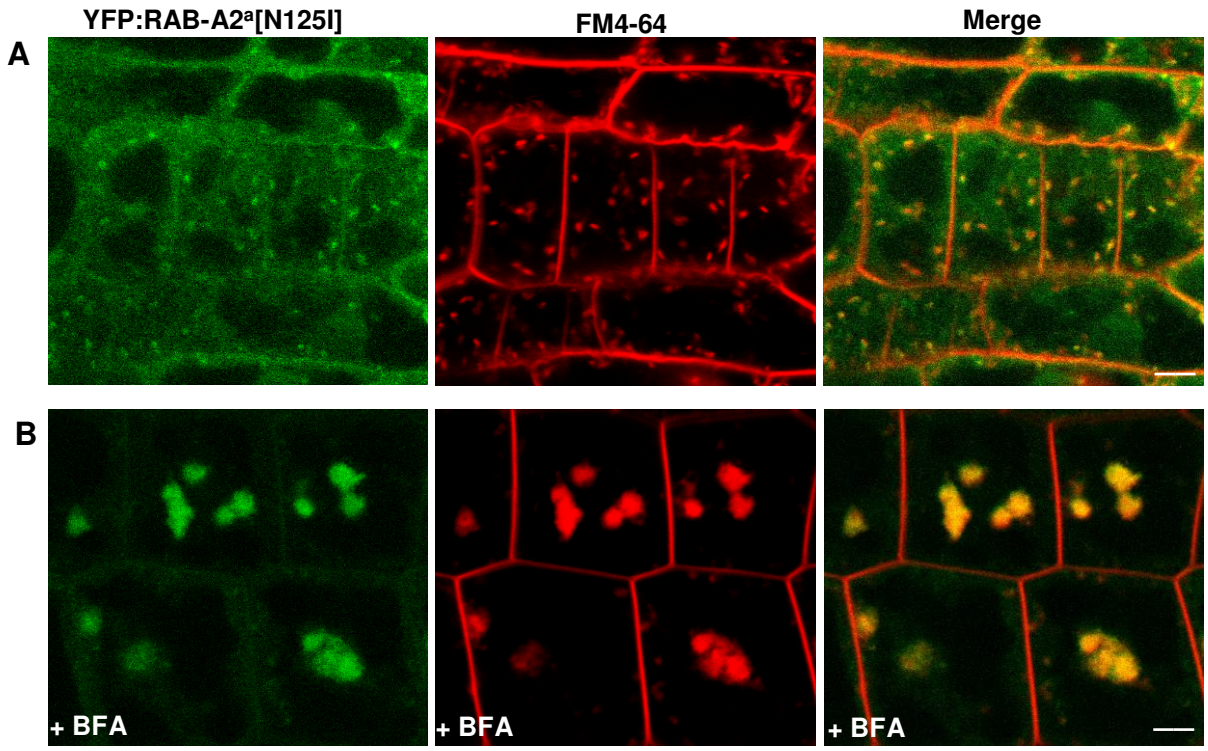
Immunolocalisation of KN (red) in *Arabidopsis* seedling roots expressing xFP:RAB-A (green) stained with DAPI (blue). Mitotic cells expressing YFP:RAB-A2^{d} (A-B), YFP:RAB-A2^{a} (C) or GFP:*Ps*RAB-A3 (D) at the prophase (A), metaphase (B & D) or anaphase (C). Enlarged images of boxed area are shown as an insert (A). White arrows: extensive overlap between xFP and KN signals. Yellow arrows: xFP signal alone. Pink arrows: KN signal alone. Substantial-colocalisation between xFP and KN is found in metaphase and anaphase. Scale bars: 5μm.



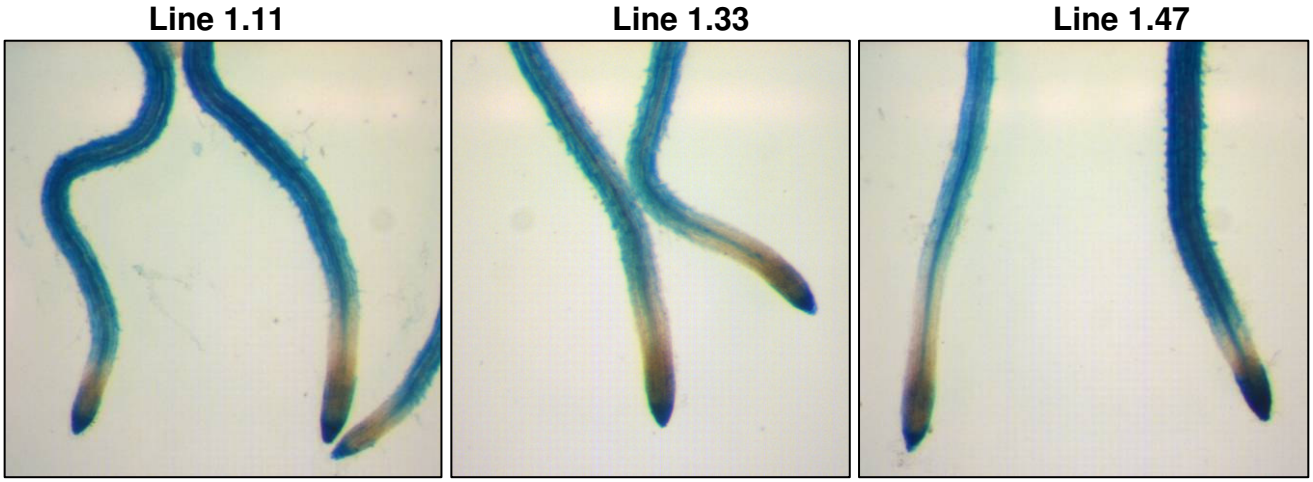
Supplemental Figure 16. YFP:RAB-A2/A3-labelled compartments and mRFP:RAB-F2^{b} compartments remain distinct during cytokinesis.
FM4-64 (red) labelling of cells expressing mRFP:RAB-F2^{b} (A-B), YFP:RAB-A2^{c} (C), YFP:RAB-A2^{b} (D) or YFP:RAB-A3 (E) (each green). YFP:RAB-A2/A3 localised to FM4-64-labelled punctate structures and cell plates in cytokinetic cells while mRFP:RAB-F2^{b} compartments were distinct from FM4-64-labelled structures. White arrows: extensive overlap between xFP and FM4-64 signals. Yellow arrows: xFP signal alone. Pink arrows: FM4-64 signal alone. Times in the brackets (middle row) are the incubation times with FM4-64. Scale bars: 5µm.



Supplemental Figure 17. Cell plate localisation is not a general feature of xFP:RAB fusions.
A. Coexpression of GFP:*Ps*RAB-A3 (green) with YFP fusions (red) to either *At*RAB-A2^{d}, *At*RAB-B1^{b} , *At*RAB-C1, *At*RAB-H1^{b} or *At*RAB-E1^{c} in root tip cells of *Arabidopsis* seedlings. YFP:RAB-B1^{b} and -C1 do not label the cell-plate but some YFP:RAB-H1^{b} and YFP:RAB-E1^{c} do
B. Immunolocalisation of KN (red) in root tip cells expressing YFP:RAB-H1^{b} or YFP:RAB-E1^{c} (green) stained with DAPI (blue). White arrows: extensive overlap between YFP:RAB and KN signals. Yellow arrows: YFP:RAB signal alone. Pink arrows: KN signal alone. Enlarged image of the box area is shown as an inset, where YFP:RAB-E1^{c} and KN localised to closely associated yet distinct structures, even though they overlapped in the cell plate.



Supplemental Figure 18. YFP:RAB-A2^a[N125I] labels FM4-64-positive endosomes. Colocalisation of YFP:RAB-A2^a[N125I] (green) and FM4-64 (red) in the absence (A) and presence (B) of brefeldin-A. The mutant protein colocalises with FM4-64-positive endosomes and BFA bodies as wild-type. Bars = 4µm.



Supplemental Figure 19. Inducible expression from the CaMV35S:LhGR activator line 4c is weaker in the meristematic region of the root.

Seedlings stained briefly (<1hr) for GUS reporter activity to show preferential staining outside the root meristem. Seedlings are from three indepenedent lines expressing wild-type *Arabidopsis* RAB-A2^{a} from pH-TOP in the 4c activator background (Craft et al., 2005) and were germinated and grown on dexamethasone-containing medium for 10 days before staining. The GUS reporter in pH-TOP is expressed from the same inducible promoter as the *RAB-A2^{a}* sequence (Craft et al., 2005). Longer staining revealed GUS activity throughout the root tip.